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Critical Temperature and Heating Time for Coagulation Damage: Implications for Interstitial Laser Coagulation (ILC) of Tumors

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Background: **Interstitial laser coagulation (ILC) is a method of local tissue destruction for solid tumors such as irresectable hepatic metastases from colorectal cancer. With the availability of new magnetic resonance (MR) techniques, which allow real time tissue temperature mapping, it is essential to know the critical temperature and exposure times leading to cell death.**
Materials and Methods/ Study Design: **Samples (8 mm^3) of solid rat tumor (CC-531, syngenic to the WAG/Rij rat strain), were warmed in tubes for four different temperatures (40, 50, 60 or 80°C) and four different exposure times (3, 6, 12, or 24 minutes). Combinations were replicated in five-fold. Cell viability was assessed with three methods: Trypan blue exclusion test in collagenase/dispase dissociated samples, NADH activity in snap frozen samples and outgrowth for 2 weeks under the renal capsule of WAG/Rij rats.**
Results: **Results of the three methods revealed that viability was not affected with heating at 40 and 50°C except for 24 minutes at 50°C. At higher temperatures cell death occurred at all exposure times.**
Conclusion: **The temperature range resulting in sufficient tissue coagulation for cell death is between 50°C and 60°C for a short duration (<3 minutes). These data can be used to achieve complete tumor destruction and minimal surrounding tissue damage during real-time MR-controlled ILC. Lasers Surg. Med. 25:257–262, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: CC-531; ILC; metastases; thermometry; thermotherapy

INTRODUCTION

Interstitial laser coagulation (ILC) is an emerging treatment modality for patients with solid lesions in parenchymal organs such as hepatic and brain tumors [1]. Upon absorption of the laser light (either Nd:YAG or Diode) several thermal effects may take place.

Coagulation is defined as the irreversible thermal damage of tissue proteins at temperatures between 55°C and 95°C. If the temperature exceeds 100°C, water vaporization followed by tissue carbonization and ablation occurs [2,3]. Protein denaturation consists of two stages: unfolding of the protein and subsequently aggregation of which the latter is irreversible [4,5]. From studies on dissolved individual proteins, it is known that there is a large variability between different proteins in the temperature at which coagulation occurs (T_c) with a range between 48°C and 90°C [6,7]. Cell viability is connected to the thermostability of several critical proteins with a T_c around

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60°C [8–10]. Formation and extent of coagulation are dependent on the time during which the temperature remains at this range. The crux of ILC is that the executed amount of protein denaturation is sufficient to result in cell death. Although it is generally assumed that in solid tissue and tumor, warmed at 60°C for several minutes, complete protein denaturation occurs, there are no supporting experimental data for ILC of tumors.

So far, exact knowledge of the critical temperature has not been relevant as temperature could not be measured precisely enough in tissue at real time. Recent advances in MR temperature assessment warrant precise temperature estimates in an unlimited number of locations and thus allow thermometry to monitor and to control ILC [11,12]. This development actualizes the question to the optimal combination of temperature and exposure time necessary for tissue coagulation in solid tumor.

MATERIALS AND METHODS

The effects of combinations of four temperatures (40, 50, 60 or 80°C) and four different exposure times (3, 6, 12, or 24 minutes) were investigated (16 groups). Combinations were replicated in five-fold. Negative control samples were stored at 4°C were used and positive controls consisted of microwave treated samples (cooking for 1 minute). Cell viability after heating was assessed with three methods [13–15]: 1) Trypan blue exclusion test in collagenase/dispase dissociated samples, 2) mitochondrial NADH activity in snap frozen samples, and 3) outgrowth for 2 weeks under the renal capsule in Wag/Rij rats (syngeneic to the used tumor).

Ether anesthesia was used for all surgical procedures. The experimental protocol adhered to rules laid down by the Dutch Animal Experimentation Act and was approved by the Committee in Animal Research of the Erasmus University Rotterdam.

Tumor Line and Sample Harvest

CC531 is a moderately differentiated, weakly immunogenic colonic adenocarcinoma induced in a WAG/Rij rat by administration of 1,2-dimethylhydrazine [16]. A cell line has been established from this carcinoma, which is maintained by serial passage after trypsinization in culture medium. CC531 tumor cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, glutamin (2 mM), and penicillin (10^5 U/L), and passaged twice weekly (all supplements Gibco, Paisley, UK). Before use, cells were trypsinized, centrifuged, and resuspended in medium. To grow solid tumors, 1×10^8 CC-531 tumor cells were injected in the right and in the left hind flanks of inbred WAG/Rij rats (Harlan-CPB, Zeist, The Netherlands). After 6 weeks tumor volume in both flanks had reached a volume of approximately 2.5 cm^3 and the mass was isolated aseptically with a scalpel from the outer membrane. Subsequently the tumor was divided into samples of approximately 8 mm^3 and immersed in medium on ice till further procedures. The necrotic center of tumor was not used for tumor sample harvest.

Heating

The obtained tumor samples were immersed per four in tubes (Eppendorf, Hamburg, Germany), filled up with 1 ml medium at 4°C. Immediately the tubes were placed in a thermostat controlled water bath [Dépex, De Bilt, The Netherlands]. From pilot studies, it was learned that 30 sec sufficed to raise the core of the samples to the temperature of the water bath [determined with four fiberoptic thermosensors (SMM interstitial probe and model 790, Fluoroptic Thermometry System, Luxtron, Santa Clara, CA) positioned 1) in the center of sample, 2) attached to the sample, 3) at the tube's wall, and 4) at the transitition medium to air, respectively]. Thereafter the randomized exposure duration was calculated. Immediately following heating the tubes were stored on ice till further processing.

Evaluation

Vitality of three of the four samples in each tube was evaluated, each with a different method:

Trypan blue exclusion. One sample was dissociated by sonication in a water bath at 37°C in medium containing 1.5 mg/ml Collagenase/Dispase (Sigma, Steinheim, Germany) [12]. After incubation in Trypan Blue solution the percentage of dead cells was assessed and expressed as those incorporating the blue pigment [17].

NADH histochemistry. Staining with nitro blue tetrazolium (NBT) can be used to stain NADH-diaphorase activity which is a measure for mitochondrial metabolism. Upon cell death the activity of NADH-diaphorase subsides [14]. Snap-frozen samples were stored at –80°C and serial sections of 8 μm were cut a cryostat. Sections were picked up onto clean glass slides. Incubation solution consisted of 9 mg NADH (Sigma) 3 ml

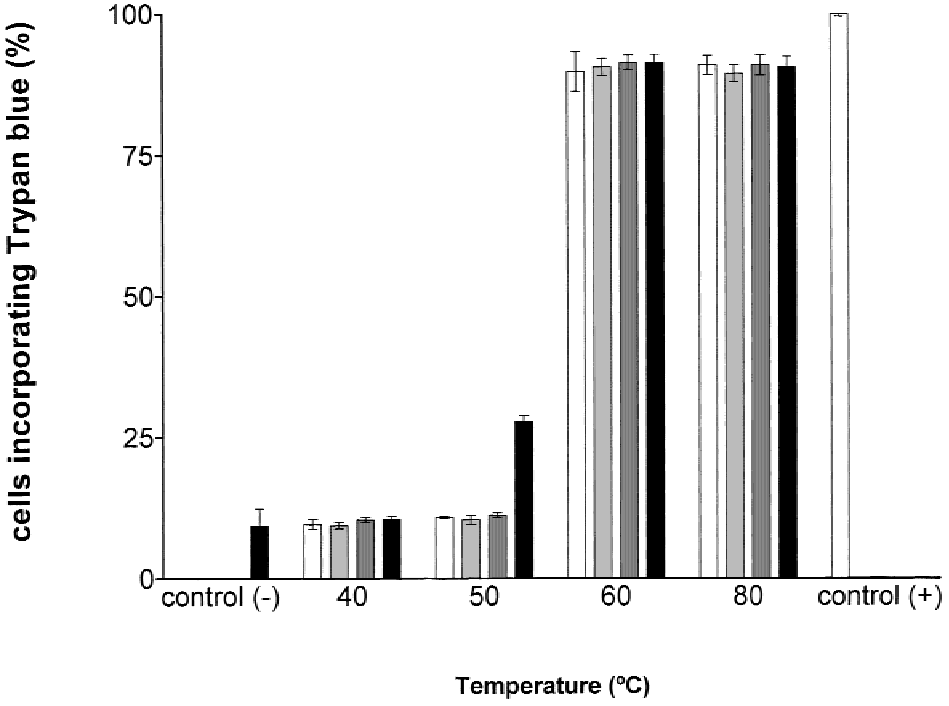

Fig. 1. Average percentage of cells incorporating Trypan blue solution. White, 3 minutes; light gray, 6 minutes; dark gray, 12 minutes; and black, 24 minutes. Bars indicate standard error of the mean and results are the mean of five experiments.

phosphate-buffered saline, 12.1 ml distilled water, and 35 mg NBT (Sigma). Sections were covered with 100 ml solution and incubated for 60 minutes at 37°C at aerobic conditions. Sections were washed with distilled water, mounted in a formaline/macrodex solution medium, and covered with a glass cover slip. With light microscopy mitochondrial activity was visible as blue staining. Proportion of staining was scored was 1, <25%; 2, 25–49%; 3, 50–74%, or 4, >75%).

Sub renal capsular assay. Individual samples were weighted. After a small midline laparotomy both poles of the left and right kidney were exposed. With microscopic vision the capsule at each pole was dissected to allow the placement of one sample at each pole. The laparotomy was closed in one layer. Two weeks after implantation all animals were sacrificed. Growth of the tumor sample was determined as the weight difference between the enucleated lump and the implanted sample.

Statistics

All numerical data are expressed as mean ± standard error of the mean (SEM) and tested for difference between the experimental groups with Student's t-test. Differences between groups associated with exposure duration or temperature of heating were analyzed using a two-way of analysis of variance test with both variables as factor [18]. Results were considered statistically significant at $P \leq 0.05$.

RESULTS

All animals tolerated the experimental procedures. Tumor take varied between the five donor rats and left and right thighs but net yield was sufficient for the experiments.

Trypan Blue Exclusion

In general, yield of dissociated cells was low, especially in the samples heated at 80°C for all exposures durations and positive controls. Per view a minimum of 1.3×10^6 cells was always visible. In Figure 1 the results are shown. Heating at 40°C at all exposure durations and at 50°C except for the longest duration (24 minutes) did not affect viability compared to negative controls. The number of viable cells after heating at 50°C for 24 minutes dropped to 73%. At 60°C and 80°C cell viability was < 12%. No statistically significant differences were found between heating at 60°C and 80°C, irrespective of exposure duration ($P = 0.78$).

NADH Histochemistry

In samples heated at 40°C for all exposure durations and at 3, 6, and 12 minutes heating at 50°C the number of cells exposing mitchondrical

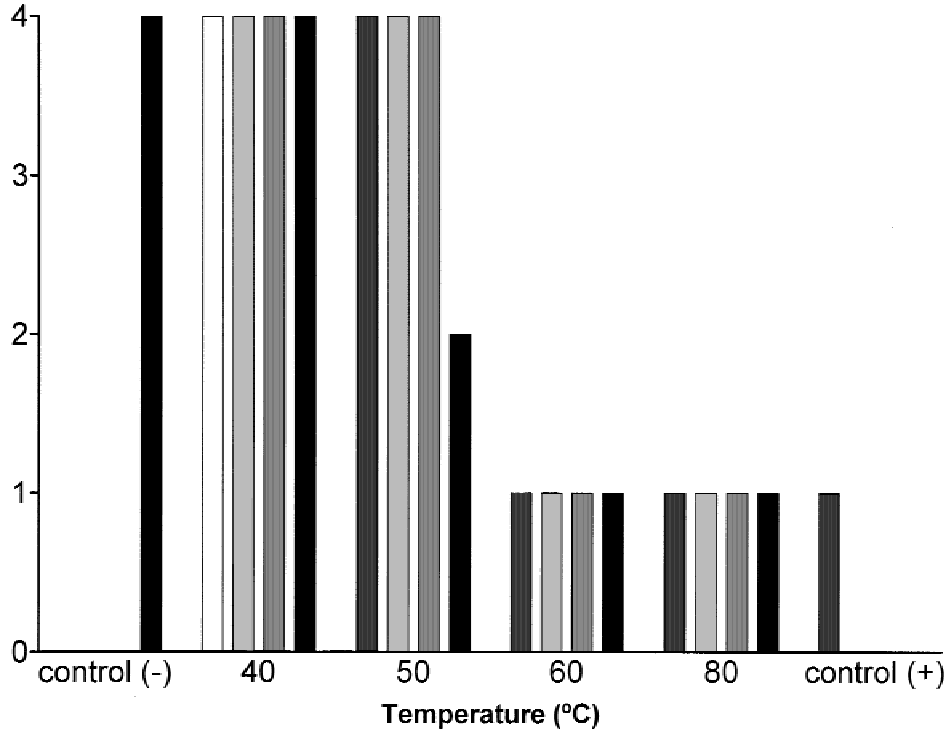

Fig. 2. Proportion of cells staining positive for mitochondrial NADH activity, scored in four groups: 1, <25%; 2, 25–49%; 3, 50–74% or 4, >75%). White, 3 minutes; light gray, 6 minutes; dark gray, 12 minutes; and black, 24 minutes. Results are the mean of five experiments.

NADH activity was always > 75%. In samples heated at 60°C and 80°C barely blue staining was detectable, indicating an absence of mitochondrial metabolism (Fig. 2).

Subrenal Capsular Assay

Median (range) sample weight prior to implantation was 51.8 g (41.3–60.9) with an even distribution between the different groups (all $F >$ 0.56). Samples heated at 40°C and 50°C showed the typical aspect of proliferating CC531 tumor. These samples had a proliferate growth and had increased in weight. Samples heated at 60°C and 80°C had a cystic appearance and did not show growth in any experiment (Fig. 3).

DISCUSSION

In this experiment the critical temperature and exposure duration for tissue coagulation and tumor death were investigated for small tumor samples. It was found that a temperature above 50°C and below 60°C, which is applied for a short duration (< 3 minutes), results in sufficient coagulation for complete cell death. These data are in line with earlier results from protein suspensions and cell solutions [8–10]. As the accuracy of noninvasive temperature measurements during ILC for tumors has increased in recent years, the present results may be used to determine the degree of coagulation at a certain point in the heated tissue.

Three established methods of assessing cellular viability and anti tumor effects were used [13–15]. Direct cell death was examined using NADH staining in snap frozen samples. This technique has been shown to be superior for this particular purpose compared to traditional assessment of morphological changes with light microscopy [13] and has been used before to investigate laser induced histological changes [14]. Membrane integrity was tested with Trypan blue exclusion in dissociated tissue samples. The crux of ILC, namely, complete cell death, was studied with outgrowth at long term [15]. The results of the three methods were concordant.

In the present study tissue samples were heated in water bath to obtain homogeneous warming. Castrèn-Person et al. [19] noted in heat sensitivity studies of cell cultures a difference in the effect between the laser-induced hyperthermia temperatures between and water bath heating at the same temperature in the control group. In agreement with the present study it was found that the largest prorportion of cell killing is executed between 49°C and 59°C. Castrèn-Person et al. [19] concluded that apart from heat induced cytotoxicity also a direct light toxicity may play a

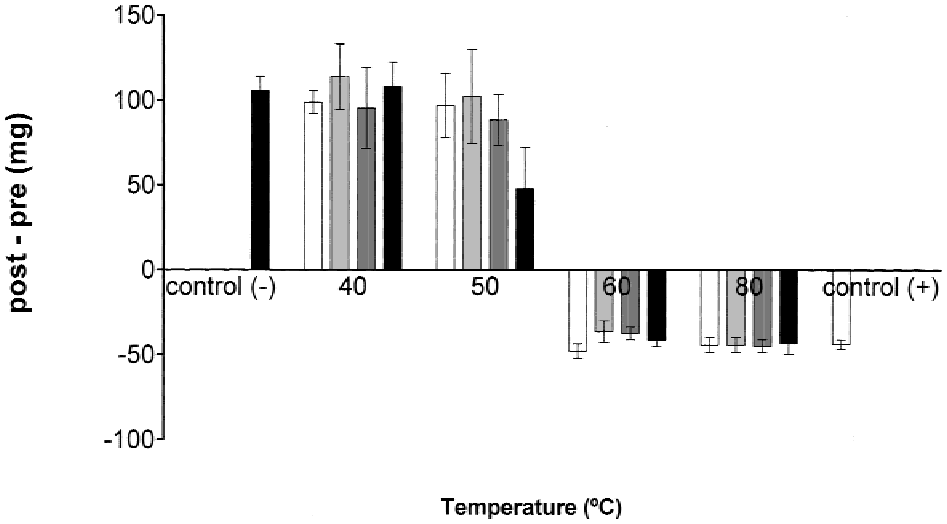

Fig. 3. Mean weight difference after 2 weeks implantation under the renal capsule (weight enucleated lump minus weight of implanted sample). White, 3 minutes; light gray, 6 minutes; dark gray, 12 minutes; and black, 24 minutes. Bars indicate standard error of the mean and results are the mean of five experiments.

role. In ILC, however, temperatures remote from the laser tip are determined by heat conduction [20]. Therefore we take the view that the present study represents the situation at locations of interest in the tumor: at the border and near important structures within the organ.

For traditional hyperthermia, aiming at temperatures between 42°C and 45°C, cellular viability of heated tissue has been investigated previously. Ikeda et al. [21] studied histological changes in canine brain after heating with times up to 60 min. A temperature of 44°C during more than 45 minutes resulted in necrosis, whereas with 42°C for 30 minutes no abnormal signs were found in tissue. The results of the latter combination are concordant to the results in the present study.

In a different setup Schulze et al. came to a very precise determination of the threshold for coagulation [22]. Using magnetic resonance imaging to monitor interstitial temperatures, the isotherm ranging between 60°C and 65°C was found to correspond to the largest dimensions of the coagulated tissue. However, as golden standard the investigators used a MRI technique of which the accuracy has not yet been established [11]. Therefore this threshold should be interpreted with caution.

In the present study, the temperature intervals were large and further studies are required for a more precise determination of the exact temperature and exposure duration at which cell death occurs. In this respect Mordon et al. [23] reported on an interesting tool. Liposomes were filled with fluorescent dyes and injected intravenously in rats prior to ILC in liver. These liposomes were pharmaceutically engineered to release the dyes with temperature increase. The amount of drug release was found to be linearly to temperature (measured with thermoprobes). Liposomes can be tailored to release their contents at any given temperature and might thus be useful to study the exact temperature at which tissue coagulation occurs. As a guide the present results can be used.

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